Development of ectomycorrhizae following inoculation of containerized Sitka and white spruce seedlings

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Received May 7, 1981[1]

Accepted October 30, 1981

SHAW, C. G., III, R. MOLINA, and J. WALDEN. 1982. Development of ectomycorrhizae following inoculation of containerized Sitka and white spruce seedlings. Can. J. For. Res. **12**: 191–195.

Containerized Sitka (*Picea sitchensis* (Bong.) Carr.) and white spruce (*P. glauca* (Moench) Voss) were inoculated at sowing with mycelial cultures of ectomycorrhizal fungi. The seedlings were grown in 66-cm^3 cells for 6 months. Sitka spruce inoculated at Corvallis, OR, with *Hebeloma crustuliniforme* (Bull. ex St. Amans) Quél, *Amanita muscaria* (L. ex Fr.) Pers. ex Hooker, or *Laccaria laccata* (Scop. ex Fr.) Berk and Br. had, respectively, 94, 92, and 99% of their short roots mycorrhizal. Seedlings colonized by *A. muscaria* were similar in size to uninoculated controls; those colonized by *H. crustuliniforme* or *L. laccata* were significantly smaller. *Tricholoma pessundatum* (Fr.) Quél. sparsely colonized 10–25% of the Sitka spruce inoculated at Corvallis. At Petersburg, AK, 93% of the Sitka spruce inoculated with *H. crustuliniforme* were colonized with 69% of their short roots forming mycorrhizae. Colonized seedlings were similar in size to controls, except for a significantly greater ($P < 0.05$) shoot/root ratio and foliar concentrations of nitrogen and phosphorus. Sitka spruce colonized at Petersburg by *Cenococcum geophilum* Fr. and maintained at the standard nursery fertilization rate had significantly fewer short roots mycorrhizal (23%) than seedlings maintained at half the standard fertilization rate (44%). White spruce were successfully colonized by *C. geophilum* and *H. crustuliniforme*. *Pisolithus tinctorius* (Pers.) Coker and Couch failed to form mycorrhizae on either spruce.

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L'inoculation d'épinettes de Sitka (*Picea sitchensis* (Bong.) Carr.) et d'épinettes blanches (*P. glauca* (Moench) Voss) croissant en contenants a été effectuée grâce à l'ensemencement de cultures mycéliennes de champignons ectomycorhiziens. Les semis se sont développés pendant 6 mois en contenants de 66 cm^3. À Corvallis, Orégon, l'épinette de Sitka inoculée avec *Hebeloma crustuliniforme* (Bull. ex St. Amans) Quél, *Amanita muscaria* (L. ex Fr.) Pers. ex Hooker ou *Laccaria laccata* (Scop. ex Fr.) Berk and Br. avait respectivement 94, 92 et 99% de ses radicelles mycorhizées. Les semis colonisés par *A. muscaria* étaient de même dimension que les témoins non-inoculés; ceux colonisés par *H. crustuliniforme* ou *L. laccata* étaient plus petits et ce, de façon significative. *Tricholoma pessundatum* (Fr.) Quél. n'a pu coloniser que 10–25% de l'épinette de Sitka inoculée à Corvallis. À Petersburg, Alaska, 93% des épinettes de Sitka ont été colonisées après inoculation avec *H. crustuliniforme* avec 69% des courtes radicelles formant des mycorhizes. Les semis colonisés étaient de dimension identique aux témoins mais avec un rapport tige/racine beaucoup plus grand ($P < 0.05$) de même que des concentrations foliaires plus élevées en azote et en phosphore. À Petersburg, l'épinette de Sitka colonisée avec *Cenococcum geophilum* Fr. et fertilisée au niveau couramment appliquée en pépinière avait des radicelles mycorhizées à un niveau significativement inférieur (23%) à celui observé chez les semis fertilisés à la moitié de ce même niveau (44%). *Cenococcum geophilum* inocula l'épinette blanche avec succès, mais *H. crustuliniforme* et *Pisolithus tinctorius* (Pers.) Coker and Couch ne purent induire la formation de mycorhizes chez l'une ou l'autre des deux espèces d'épinettes.

[Traduit par le journal]

Introduction

Shaw and Molina (1980) recently reported the first successful establishment of selected ectomycorrhizal fungi on roots of container-grown seedlings of Sitka spruce (*Picea sitchensis*) (Bong.) Carr.). In that study *Cenococcum geophilum* Fr. and *Laccaria laccata*

[1]Revised manuscript received October 22, 1981.

(Scop. ex Fr.) Berk and Br. consistently formed ectomycorrhizae on 80–100% of the inoculated seedlings.

Trappe (1977) stressed the need for testing a wide array of fungi prior to selection of beneficial strains for wide-scale nursery inoculations. Use of isolates indigenous to the outplanting locale is significant in this regard. Therefore, in the autumn of 1978, we isolated several suspected ectomycorrhizal fungi from sporophores collected beneath Sitka spruce growing in southeast Alaska.

The objective of this study was to determine if suspected ectomycorrhizal fungi indigenous to southeast Alaska could be established through controlled inoculations on containerized seedlings of Sitka and white spruce (*Picea glauca* (Moench) Voss). Effects of fertilization rates on development of Sitka spruce seedlings inoculated with *C. geophilum* or *L. laccata* were also examined.

Methods

Preparation of inoculum and soil mixture

Fungus inocula were prepared at the Forestry Sciences Laboratory in Corvallis, OR, by procedures similar to those described by Marx and Bryan (1975) and Molina (1979). After leaching, inocula for use in Alaska were placed in plastic bags, cooled to 5°C, airfreighted in an ice chest to Juneau, and stored at 5°C until used (up to 10 days). Unfortunately, the ice chest was delayed for 3–4 days during transit to Alaska.

Inoculum was thoroughly mixed at sowing time (May 1979) with a nonsterilized commercial peat–vermiculite potting mixture at a ratio of 1 part inoculum to 7 parts potting mixture. Seedling cells (66 cm^3 Ray Leach® cells)[2] were filled firm with this medium, sown with three Sitka or white spruce seeds each, and covered with perlite. Sitka spruce seed came from Chichagof Island, AK, and white spruce from Fairbanks, AK. Cells were later thinned to one seedling each. Seedlings were reared in greenhouses at Petersburg, AK, and Corvallis, OR, and in an environmental growth chamber at Juneau, AK.

Experimental design

At Petersburg one group of Sitka spruce was arranged in a randomized block design: five treatments (four fungi and a control), 20 trees per treatment with four replications (400 total seedlings). Cells were inoculated with pure cultures of either *Amanita muscaria* (L. ex Fr.) Pers. ex Hooker (S434),[3] *Tricholoma pessundatum* (Fr.) Quél. (S-452), *Hebeloma crustuliniforme* (Bull. ex St. Amans) Quél. (S-460), or *Pisolithus tinctorius* (Pers.) Coker and Couch (S-471). Control seedlings received no fungus inoculum. The *P. tinctorius* isolate originated in Georgia; all other isolates came from southeast Alaska.

In Petersburg an additional 160 Sitka spruce were inoculated with *C. geophilum* (A-176) and 160 with *L. laccata* (S-238). These seedlings were divided into two groups of 160 seedlings, 80 for each fungal treatment. One group received fertilization at the standard rate for the Petersburg nursery and the other group at 50% of this rate, as described later. Both fungal isolates had performed well in previous inoculations of Sitka spruce (Shaw and Molina 1980).

Sitka spruce were arranged at Corvallis in a randomized-block design: six treatments (five fungi and a control), 20 trees per treatment, with four replications (480 total seedlings). Treatments were the same as those used in the randomized-block experiment at Petersburg with an additional inoculation using *L. laccata* (S-238) and *C. geophilum* (A-176) mixed 50–50.

White spruce were sown at Juneau and Petersburg. At Petersburg, a randomized-block design was used: five treatments (four inoculations and a control), 20 trees per treatment, and four replications (400 total seedlings). Cells were inoculated with pure cultures of either *C. geophilum* (A-176), *L. laccata* (S-238), *C. geophilum* (A-176) and *L. laccata* (S-238) in a 50–50 mixture, or a commercial preparation of *P. tinctorius*. Control seedlings received no inoculum. In Juneau 20 or more cells were inoculated with one of the following: *A. muscaria* (S-454), *T. pessundatum* (S-452), *H. crustuliniforme* (S-460), or *P. tinctorius* (S-471). There was also a no-inoculum control. Juneau seedlings were used only to ascertain the presence or absence of mycorrhizae.

Seedling rearing conditions

Seedlings in Petersburg received natural light supplemented with incandescent light at 215–430 lx for a 5-min interval during every half hour of natural darkness. Air temperatures ranged from 18 to 24°C. Trees were watered by hand or an overhead boom each or every other day. In this randomized-block experiment, a commercial preparation of soluble 20-19-18 (N-P-K) fertilizer was applied weekly after the 1st month at a final rate of 61 g per 10 m^2 of bench area (4 oz per 100 ft^2). This level of fertilization was also used for the white spruce at Petersburg and represented the low fertilizer rate for Sitka spruce inoculated at Petersburg with *C. geophilum* or *L. laccata*. Sitka spruce fertilized at the standard nursery rate received 122 g of 20-19-18 (N-P-K) per 10 m^2 of bench space (8 oz. per 100 ft^2).

In Corvallis seedlings were reared under a 16-h photoperiod with air temperatures ranging from 15 to 27°C. Trees were watered with an automatic misting system and fertilized twice monthly by hand with approximately 3.1 mg of solubilized 20-19-18 (N-P-K) per seedling.

In Juneau, seedlings were reared under a 16- to 18-h photoperiod of approximately 1000 lx with air temperatures varying from 15 to 27°C. A solubilized 20-19-18 (N-P-K) fertilizer, mixed at 0.1 g/L of water, was applied to the 0.28-m^2 chamber area once a week after the 1st month. At all three locations, fertilizer was amended with trace elements and iron chelate. Water, temperature, and lighting were reduced in October to induce bud set. Seedlings were harvested in December.

Sampling and statistical analysis

After harvest, each root system was gently washed free of

[2]The use of trade, firm, or corporation names does not constitute an official endorsement by the Canadian Journal of Forest Research or the U.S. Department of Agriculture.

[3]Reference numbers for the PNW culture collection maintained at the Forestry Sciences Laboratory, Corvallis.

potting mixture and examined microscopically for mycorrhizae. From each treatment replication in the randomized-block experiments, 10 seedlings showing successful inoculation and no contaminant mycorrhizae were selected at random. Degree of mycorrhizal formation was calculated by removing three or four large lateral roots and classifying each short root thereon as mycorrhizal or not. One hundred or more short roots on each seedling were examined and classified. Height, root-collar diameter, and ovendry weights of tops and roots were also recorded. Within each area, results were subjected to analyses of variance and differences between treatment means were compared with Tukey tests.

All significant differences were judged at $P < 0.05$. At Petersburg, control seedlings and those with mycorrhizae were analysed for concentrations of foliar phosphorus (P) and nitrogen (N). Foliage from the 10 seedlings sampled in each treatment replication in the randomized-block experiment were pooled and ground with a mortar and pestle, and a subsample was drawn from each seedling group. These samples were analysed for P by the molybdate blue colormetric procedure following a nitric–perchloric acid digestion and for N by a micro-Kjeldahl procedure. Data for control seedlings and those with mycorrhizae were paired by replication and compared with a paired Student's *t*-test (3 df; $P < 0.05$).

Half of the 80 seedlings inoculated with *C. geophilum* at Petersburg and fertilized at each level were randomly selected and measured as above. Data for seedling growth at the two fertilization levels and the relative number of shoot roots colonized by *C. geophilum* were compared by Student's *t*-test at $P < 0.05$. The seedlings inoculated with *L. laccata* at Petersburg and similarly maintained were not analysed because mycorrhizae failed to form.

Results

At Petersburg and Corvallis, respectively, 93 and 100% of the Sitka spruce inoculated with *H. crustuliniforme* formed mycorrhizae. In addition, 100% of the Sitka spruce inoculated in Corvallis with *A. muscaria*, 100% inoculated with the *L. laccata* − *C. geophilum* mixture, and 10–25% inoculated with *T. pessundatum* formed mycorrhizae. In the mixed-inoculum treatment at Corvallis, *L. laccata* formed abundant mycorrhizae but no *C. geophilum* mycorrhizae developed. At Petersburg, *A. muscaria* and *T. pessundatum* failed to form mycorrhizae. *Laccaria laccata* was not used in the randomized-block experiment.

Laccaria laccata, however, failed to form mycorrhizae at Petersburg on Sitka spruce fertilized at the standard or low rate. Contrariwise, *C. geophilum* formed mycorrhizae on 100% of the seedlings at both fertilization levels. On seedlings colonized by *C. geophilum*, no significant differences occurred in seedling height, diameter, shoot weight, or root weight, between the two levels of fertilization. Shoot weight/root weight ratio, however, was significantly less for seedlings fertilized at the low rate (2.55 vs. 3.12). Also, a significantly greater percentage of short roots (44 vs. 23%) were colonized on seedlings receiving the low fertilizer treatment. We did not have controls at the two fertilization levels to provide growth comparisons between colonized and uncolonized seedlings.

The percentage of short roots that were mycorrhizal on Sitka spruce seedlings successfully colonized by *H. crustuliniforme* was 94 and 69% at Corvallis and Petersburg, respectively, and 72 and 99%, respectively, for *A. muscaria* and *L. laccata* at Corvallis. Seedlings colonized by *H. crustuliniforme* or *L. laccata* at Corvallis were significantly smaller than controls in height, diameter, and shoot weight (Table 1). Root weight for seedlings colonized by *H. crustuliniforme* was also significantly less than for controls (Table 1). In contrast, seedlings colonized by *A. muscaria* did not differ significantly from control seedlings in these growth measurements. Although seedlings colonized by *L. laccata* and *H. crustuliniforme* had larger mean shoot/root ratios than controls, the differences were not significant (Table 1). Seedling growth and percentage of short roots colonized by *T. pessundatum* at Corvallis were not calculated because the percent of seedlings on which mycorrhizae formed was relatively low. Mycorrhizal formation on these seedlings was generally sparse and localized to small portions of the root system.

At Petersburg, the shoot/root ratio of Sitka spruce colonized by *H. crustuliniforme* was significantly greater than controls (2.32 vs. 1.96); the only growth measurement showing a significant difference. In addition, seedlings colonized by *H. crustuliniforme* had significantly greater percents of P and N in the foliage (0.24 and 1.7%, respectively) than did control seedlings (0.19 and 1.3%, respectively).

At Petersburg, mycorrhizae formed on 99% of the white spruce inoculated with *C. geophilum* alone, but none formed on seedlings inoculated with *P. tinctorius* or *L. laccata* alone. No *L. laccata* mycorrhizae formed on seedlings inoculated with the *L. laccata* − *C. geophilum* mixture, but 80% of those seedlings developed *C. geophilum* mycorrhizae. On white spruce inoculated with *C. geophilum* alone, 18% of the short roots were mycorrhizal, but the seedlings did not differ significantly from controls in any measure of growth. At Juneau, *H. crustuliniforme* formed mycorrhizae on 100% of the inoculated white spruce, but *A. muscaria*, *T. pessundatum*, and *P. tinctorius* failed to form mycorrhizae.

Discussion

Even though all of the Sitka spruce inoculated in Corvallis with *A. muscaria* and *L. laccata* formed mycorrhizae, both fungi failed to develop mycorrhizae at either location in Alaska. This discrepancy may be attributable to the delay in air transport of inocula from Oregon to Alaska, during which inocula might have

TABLE. 1. Growth and mycorrhizal formation for Sitka and seedlings inoculated at Corvallis, OR, with various mycorrhizal fungi*

Treatment	Shoot height (g)	Shoot diameter (mm)	Shoot weight (g)	Root weight (g)	Shoot weight root weight ratio	% short roots mycorrhizal
Control	16.6*a*†	2.15*a*	0.79*a*	0.39*a*	2.08	0
Hebeloma crustuliniforme	14.5*ab*	1.83*b*	0.50*b*	0.24*b*	2.43	94*a*
Amanita muscaria	16.8*a*	2.11*a*	0.77*a*	0.40*a*	2.02	72*b*
Laccaria laccata	13.1*b*	1.90*b*	0.62*c*	0.31*ab*	2.31	99*a*

*Mean of 10 seedlings showing no contaminant mycorrhizae per replication; 40 total for each treatment. Forty seedlings were randomly selected from each fertilizer treatment.

†Values not sharing a common letter are significantly different ($P < 0.05$) by Tukey tests or Student's *t*-test for fertilization treatments. When no letters follow a group of values significant differences did not occur. Control seedlings were not used in analysis for percent of mycorrhizal short roots.

been exposed to adverse temperatures. This may have differentially affected the viability of the various species. Inoculum of *L. laccata* was viable after transport to Alaska in a previous inoculation (Shaw and Molina 1980) but no delay occurred during that transport.

Failure of *C. geophilum* to form mycorrhizae with Sitka spruce inoculated at Corvallis with the *C. geophilum* − *L. laccata* mixture is not attributed to poor inoculum as the same *C. geophilum* inoculum was viable in Alaska. Rather, we suggest that *L. laccata* more quickly colonized short roots as they formed, thus occupying potential colonization sites before *C. geophilum* could form mycorrhizae. This suggestion is supported by the observation that *L. laccata* mycelium developed more rapidly and more thoroughly colonized the vermiculite substrate during incubation of inoculum, creating a greater inoculum density for *L. laccata* than for *C. geophilum*. Also *L. laccata* has generally colonized more short roots than *C. geophilum* in previous single isolate inoculations (Shaw and Molina 1980; Molina 1980). On *Pinus strobus* L. the rate of mycorrhizal formation by *C. geophilum* was considerably slower than that of *P. tinctorius* (Fortin and Piche 1980), suggesting that *C. geophilum* may be a relatively slow mycorrhizal former under these conditions and thus be at a disadvantage in any mixed-species inoculations. Trappe (1962) reported that *C. geophilum* is a predominant mycorrhizal fungus in the field only where environmental conditions limit growth of other fungi.

Failure of *P. tinctorius* to form mycorrhizae corresponds to our previous results with Sitka spruce (Shaw and Molina 1980). *Pisolithus tinctorius* has been successfully inoculated onto the roots of several conifers (Marx 1977; Molina 1979) and will colonize Sitka spruce in pure culture (R. Molina, unpublished data). The isolate of *P. tinctorius* that we used, however, does not appear well suited for inoculation of containerized Sitka spruce seedlings by our methodology.

Results of fertilization rate on mycorrhizal formation by *C. geophilum* must be viewed as preliminary, but they suggest that standard nursery fertilization schedules for containerized Sitka spruce may not be conducive to mycorrhizal formation. Similar results have been noted for formation of mycorrhizae by other fungi on other hosts (Marx and Barnett 1974; Ruehle and Marx 1977; J. M. Trappe and R. Molina, unpublished data).

Ectomycorrhizal inoculation of containerized seedlings rarely aids growth in the nursery (Marx and Barnett 1974; Molina 1980). At Corvallis, mycorrhizal formation by *L. laccata* and *H. crustuliniforme* was associated with reduced seedling growth; in contrast, seedlings colonized by *A. muscaria* were the same size as controls. Apparently formation of mycorrhizae by *A. muscaria* requires less host photosynthate than does formation of mycorrhizae by *L. laccata* or *H. crustuliniforme*. We suspect that the prolific mycelial colonization of the potting medium by *L. laccata* and *H. crustuliniforme* and the production of sporophores by *L. laccata* (Shaw and Molina 1980) reduces host photosynthates available for seedling growth. Hence, seedlings colonized by *L. laccata* or *H. crustuliniforme* are generally smaller than controls. Slightly smaller seedlings successfully colonized with beneficial fungi, however, may upon planting outperform larger, nonmycorrhizal seedlings, especially if the mycorrhizal seedlings have a more favorable nutrient status at the time of outplanting. We are currently testing this hypothesis.

White spruce is the predominant conifer over several million acres (1 acre = 0.404 685 6 ha) of temperate forest land in central Canada and interior Alaska. Both the State of Alaska and the Canadian Forest Service

have containerized nursery facilities for rearing white spruce seedlings. Trappe (1964) reported *C. geophilum* as forming ectomycorrhizae with white spruce and J. Dangerfield (unpublished data) has inoculated white spruce with *P. tinctorius*. Although many potential mycorrhizal fungi sporulate in association with white spruce, we have found no other experimental confirmation of additional white spruce associates in the literature.

FORTIN, J. A., and Y. PICHE. 1980. Technique for the observation of early morphological changes during ectomycorrhizae formation. Can. J. Bot. **58**: 361–365.

MARX, D. H. 1977. Tree host range and world distribution of the ectomycorrhizal fungus *Pisolithus tinctorius*. Can. J. Microbiol. **23**: 217–223.

MARX, D. H., and J. P. BARNETT. 1974. Mycorrhizae and containerized forest tree seedling. *In* Proceedings of the North American containerized forest tree seedling symposium. *Edited by* R. W. Tinus, W. I. Stein, and W. E. Balmer. Great Plains Agriculture Council Publication No. 68. pp. 85–92.

MARX, D. H., and W. C. BRYAN. 1975. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. For. Sci. **21**: 245–254.

MOLINA, R. 1979. Ectomycorrhizal inoculations of containerized Douglas-fir and lodgepole pine seedlings with six isolates of *Pisolithus tinctorius*. For. Sci. **25**: 585–590.

——— 1980. Ectomycorrhizal inoculation of containerized western conifer seedlings. U.S. Dep. Agric. For. Serv. Res. Note PNW-357.

RUEHLE, J. L., and D. H. MARX. 1977. Developing ectomycorrhizae on containerized pine seedlings. U.S. For. Serv. Res. Note SE-242.

SHAW, C. G., III, and R. MOLINA. 1980. Formation of ectomycorrhizae following inoculation of containerized Sitka spruce seedlings. U.S. Dep. Agric. For. Serv. Res. Note PNW-351.

TRAPPE, J. M. 1962. *Cenococcum graniforme*—its distribution, ecology, mycorrhiza formation, and inherent variation. Ph.D. thesis, University of Washington, Seattle.

——— 1964. Mycorrhizal hosts and distribution of *Cenococcum graniforme*. Lloydia, **27**: 100–106.

——— 1977. Considerations in selection of fungi for ectomycorrhizal inoculation in nurseries. Annu. Rev. Phytopathol. **15**: 203–222.